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Application of:	Ensoli	Confirmation No.:	9400
Application No.:	09/555,534	Art Unit:	1648
Filed:	May 31, 2000	Examiner:	Stucker, Jeffrey J.
For:	HIV TAT, OR DERIVATIVES THEREOF FOR PROPHYLACTIC AND THERAPEUTIC VACCINATION	Attorney Docket No.:	11340-003-999 (formerly 204.610)

SECOND DECLARATION OF SHAYNE GAD, Ph.D. UNDER 37 C.F.R. § 1.132

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, SHAYNE GAD, Ph.D., do declare as follows:

1. I have over 29 years experience as a toxicologist and consultant in the research and development of products in, *inter alia*, the biotechnology and pharmaceutical industries. I have a Ph.D. in pharmacology and toxicology from University of Texas at Austin and am a Diplomat of American Board of Toxicology (D.A.B.T.) and a fellow of the Academy of Toxicological Sciences (A.T.S.). I am currently principal of Gad Consulting Services, in which capacity I assist clients in evaluating the safety of biotechnology and pharmaceutical products, obtaining regulatory approval and with respect to other compliance issues before a number of United States and foreign regulatory bodies. My education and experience are summarized on my Curriculum Vitae, which is attached hereto as Exhibit 1.

2. I have been hired as a consultant by Applicant's representatives, and asked to evaluate whether the compositions of HIV-1 Tat protein ("Tat") produced according to the purification methods described by Chang *et al.*, 1997, AIDS 11:1421-1431 ("the Chang reference;" attached hereto as Exhibit 2) would be pharmaceutically acceptable for administration to a human. In my opinion, the phrase "pharmaceutically acceptable for administration to a human" when used to describe a composition means that the composition

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is sufficiently safe for administration to human patients using the criteria for safety defined by regulatory agencies such as the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA). Thus, the composition does not contain ingredients that would be recognized as prohibited for human administration by such regulatory agencies. As discussed in detail herein and for the reasons set forth below, and based on certain assumptions discussed below, it is my judgment and opinion that the Tat compositions resulting from the two purification methods described on page 1424 of the Chang reference would not be pharmaceutically acceptable for administration to a human.

First Chang Reference Tat Purification Method

3. I first address the composition produced by the method described on page 1424 of the Chang reference under the heading "Tat protein and anti-Tat antibody." The Chang reference states that the procedure involves expression of the protein in *E. coli* and then isolation of Tat through rounds of high-pressure liquid chromatography (HPLC) and ion-exchange chromatography, which the Chang reference indicates is performed as described in two earlier references, reference 18, which is Ensoli *et al.*, 1993, J. Virology 67: 277-287 (attached as Exhibit 3), and reference 44, which is Bohan *et al.*, 1992, Gene Expression 2:391-407 (attached as Exhibit 4). The relevant passage of Ensoli *et al.*, reference 18, is on page 278, column 1. The relevant passage of Bohan *et al.*, reference 44, is in the paragraph spanning pages 393 to 395, which indicates that the HPLC is reverse phase HPLC. The Tat is then lyophilized and, prior to use, resuspended in a buffer of PBS containing 0.1% Bovine Serum Albumin (BSA) and 0.1 mM dithiothreitol (DTT). In the procedures involving use of Tat reported in the Chang reference, plasticware was rinsed in either PBS-BSA buffer or 10% Fetal Calf Serum (FCS)-RPMI.

4. The Chang reference and its cited references 18 and 44 do not disclose the solvent used for HPLC. I am informed that a solvent commonly used for reverse phase HPLC is acetonitrile, usually also containing trifluoroacetic acid (TFA). I am also informed that the HPLC step would not be performed on the *E. coli* extract but, rather, would follow the ion-exchange chromatography in the purification protocol. Thus, if a solvent containing acetonitrile and TFA were used, and based upon the foregoing understanding regarding order of the steps, one would expect acetonitrile and TFA to contaminate the resulting Tat composition. Acetonitrile and TFA would be considered process impurities.

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5. Acetonitrile and TFA are each very toxic both acutely and upon repeat exposure and both are mutagens (*see, e.g.*, Ahmed *et al.*, 1992, Pharmacol. Toxicol. 70:322-330, attached as Exhibit 5; Robles *et al.*, 2005, Acetonitrile, in *Encyclopedia of Toxicology, Second Edition*, (Wexler, ed.) Elsevier, Philadelphia, PA, pp. 28-30, attached as Exhibit 6; Toxsys database Record No. AL7700000 for Acetonitrile, attached as Exhibit 7; and Toxsys database Record No. AJ9625000 for Acetic acid, trifluoro, attached as Exhibit 8). Levels of acetonitrile in therapeutics are very restricted. TFA is not recognized as an allowed pharmaceutical ingredient in any form. In addition, since TFA is a mutagen and a teratogen, it would be strenuously avoided in the production process of any therapeutic. As such, these solvents should be avoided in any production process for a therapeutic and would not be used in any production process where they might appear as a detectable impurity. Accordingly, compositions of Tat purified using acetonitrile and TFA as solvents would not be pharmaceutically acceptable for administration to a human.

Second Chang Reference Tat Purification Method

6. Second, I have also considered the method for purifying Tat described in the Chang reference on page 1424 in the paragraph spanning the two columns under the heading "Purification of recombinant Tat protein by heparin affinity chromatography." In this method, *E. coli* cells expressing Tat were sonicated in lysis buffer and the resulting lysate clarified by centrifugation, the supernatant was incubated with heparin-Sepharose prewashed with lysis buffer, and the supernatant was fractionated by heparin-Sepharose chromatography, washing the column with lysis buffer, and eluting the Tat from the column with the same lysis buffer containing 2 M NaCl. The column fractions containing the purified Tat are the final product of the purification method and contain the lysis buffer. The passage indicates that the lysis buffer contains 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Since the final step of the purification contains the 0.2 mM PMSF, it is not just a process impurity, but a component of the Tat composition.

7. PMSF, like acetonitrile and TFA, is very toxic (*see, e.g.*, Gomez-Cambronero *et al.*, 1989, In. Arch. Allergy Appl. Immunol. 4:362-368, attached as Exhibit 9; Lotti *et al.*, 1991, Toxicol. Appl. Pharmacol. 108:234-241, attached as Exhibit 10; and Massicotte *et al.*, 1999, Neurotoxicology 20:749-759, attached as Exhibit 11). PMSF is not allowed as a pharmaceutical ingredient of any form and would not even be allowed in any process where it could be detected as an impurity. Since, in this second purification method disclosed by the

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Chang reference, PMSF is an actual component of the Tat composition, this composition would not be a composition that is pharmaceutically acceptable for administration to humans.

Conclusion

8. If a solvent containing acetonitrile and TFA were used in the first purification method of the Chang reference, based upon the resulting presence of acetonitrile and TFA as process impurities in the composition, I conclude that the first purification method of Chang would not result in a Tat composition that is pharmaceutically acceptable for administration to a human. Based upon the presence of PMSF as a component of the composition resulting from the second purification method of the Chang reference, I conclude that the second purification method of Chang does not result in a Tat composition that is pharmaceutically acceptable for administration to a human.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that I make these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and any patent issuing thereon.

Date: June 14, 2006

Shayne Gad, Ph.D.